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BY: Nelene Galal

DATE: October 24 2001

PATENT

DEC 1 2 2001 (S)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application

of Ian Hector Frazer et al.

Conf. No.:

4929

Appln. No.:

09/900,345 /

Filed:

July 6, 2001

For:

METHOD AND POLYNUCLEOTIDES

: Attorney Docket

FOR DETERMINING TRANSLATIONAL

: No. 10338-5 US

EFFICIENCY OF A CODON

(2423066/VPA)

CLAIM OF FOREIGN PRIORITY AND TRANSMITTAL OF PRIORITY DOCUMENT

Applicants hereby confirm their claim of the right of foreign priority under 35 U.S.C. Section 119 for the above-identified patent application. The claim of foreign priority is based upon Application No. PP 8078, filed in Australia on January 8, 1999, and International Application No. PCT/AU00/00008, filed in Australian on January 7, 2000, and the benefit of those dates is claimed.

Submitted herewith are certified copies of both Australian Applications identified in the paragraph above. It is submitted that these documents complete the requirements of 35 U.S.C. Section 119, and benefit of the foreign priority is respectfully requested.

Respectfully submitted,

IAN HECTOR FRAZER ET AL.

7.24, 200(By

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ASN/hg/Encls.

Attorney for Applicant

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Patent Office Canberra

I, GAYE TURNER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 8078 for a patent by THE UNIVERSITY OF QUEENSLAND filed on 08 January 1999.

WITNESS my hand this Fifth day of October 2001

GAYE TURNER

TEAM LEADER EXAMINATION

SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "CODON UTILIZATION"

The invention is described in the following statement:

TITLE

"CODON UTILIZATION"

5 FIELD OF THE INVENTION

INVENTION relates generally to gene expression and in particular, а method and polynucleotides for determining codon utilization in one or more cells or tissues of an organism. More particularly, the method and polynucleotides of the invention are concerned with ascertaining preferences in cells or tissues for purposes modifying the translational efficiency of proteinencoding polynucleotides in those cells or tissues.

BACKGROUND OF THE INVENTION

It is well known that a "triplet" codon of four possible nucleotide bases can exist in 64 variant These forms provide the message for only 20 forms. different amino acids (as well as translation initiation and termination) and this means that some amino acids can be encoded by more than one codon. this context, some amino acids have as many as six "redundant", alternative codons while some others have a single, required codon.

For reasons not completely understood, codon utilization is highly biased in that alternative codons are not at all uniformly present in the endogenous DNA of differing cell types. In this regard, there appears to exist a variable natural hierarchy of "preference" for certain codons between different cell types or between different organisms.

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Codon usage patterns have been shown correlate with relative abundance of isoaccepting transfer RNA (iso-tRNA) species, and with encoding proteins of high versus low abundance. Moreover, the present inventors recently discovered that the intracellular abundance of different isotRNAs varies in different cells or tissues of a single multi-cellular organism (see copending International Application No. PCT/AU98/00530).

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implications of codon preference The phenomena on gene expression are manifest in that affect the translational can these phenomena efficiency of messenger RNA (mRNA). In this regard, it is widely known that translation of "rare codons", for which the corresponding iso-tRNA is in relatively low abundance, may cause a ribosome to pause during translation which can lead to a failure to complete a chain and an uncoupling polypeptide nascent transcription and translation.

A primary goal in recombinant research is to provide transgenic organisms which express a foreign gene in an amount sufficient to confer the desired phenotype to the organism. However, expression of the foreign gene may be severely impeded if a particular host cell of the organism or the organism itself has a low abundance of iso-tRNAs corresponding to one or more codons of the foreign gene. Accordingly, a major aim of investigators in this field is to first ascertain the codon preference for particular cells or tissues in which a foreign gene is to be expressed, and subsequently alter the codon composition of the foreign gene for optimized expression in those cells or tissues.

Codon preference may be determined simply by analyzing the frequency at which codons are used by genes expressed by a particular cell or tissue or by a

plurality of cells or tissues of a given organism. Codon frequency tables as well as suitable methods for determining frequency of codon usage in an organism are described, for example, in an article by Sharp et al (1988, Nucleic Acids Res. 16 8207-8211). The relative level of gene expression (e.g., detectable protein expression versus no detectable protein expression) can provide an indirect measure of the relative abundance of specific iso-tRNAs expressed in different cells or tissues.

Alternatively, codon preference may be determined by measuring the relative intracellular abundance of different iso-tRNA species. For example, reference may be made to copending International Application No. PCT/AU98/00530 which describes a method that utilizes labeled oligonucleotides specific for different iso-tRNAs to probe an RNA extract prepared from a particular cell or tissue type.

The above methods provide useful indirect evidence for determining codon preference. However, such indirect evidence may not provide an accurate indication of the translational efficiency of a given codon. Accordingly, there is a need to provide a method which more directly ascertains the translational efficiency of a codon in a cell or tissue.

OBJECT OF THE INVENTION

is therefore an object of the present 30 invention to provide a method for determining codon tissues which method in cells orpreference disadvantages of the some ameliorates at least associated with the prior art.

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SUMMARY OF THE INVENTION

Accordingly, in one aspect of the invention, there is provided a method for determining relative preference for a codon in at least one cell or tissue type, said method including the steps of:-

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- (A) introducing into said at least one cell or tissue type a synthetic construct comprising a reporter polynucleotide fused in frame with a tandem repeat of identical codons corresponding to said codon under test, wherein said reporter polynucleotide encodes a reporter protein, and wherein said synthetic construct is operably linked to one or more regulatory nucleotide sequences;
- (B) expressing said synthetic construct in said at least one cell or tissue type; and
 - (C) measuring activity associated with said reporter protein in said at least one cell or tissue type to thereby determine the relative preference for said codon.

Preferably, the method is further characterized by the steps of:-

- (i) introducing into another of said at least one cell or tissue type a different synthetic construct having said reporter polynucleotide fused in frame with a tandem repeat of identical codons corresponding to a different codon under test, wherein said different codon is synonymous with said first-mentioned codon, and wherein said different synthetic construct is operably linked to one or more regulatory nucleotide sequences;
- (ii) expressing said different synthetic construct in said another of said at least one cell or tissue type;

- (iii) measuring activity associated with said reporter protein in said another of said at least one cell or tissue type; and
- (iv) comparing the respective activities associated with said reporter protein from said synthetic constructs to thereby determine the preference for said first-mentioned codon relative to the preference for said different codon.

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Preferably, step (A) of the above method is further characterized by the steps of:-

- (a) introducing the synthetic construct into a progenitor cell or tissue of said at least one cell or tissue type; and
- (b) generating said at least one cell or 15 tissue type from said progenitor cell or tissue;

wherein said at least one cell or tissue type contains said synthetic construct.

Suitably, the method is further characterized by the steps of:-

- 20 (1) introducing the synthetic construct into a progenitor cell or tissue of said at least one cell or tissue type; and
 - (2) growing an organism or part thereof from said progenitor cell or tissue;
- wherein said organism comprises said at least one cell or tissue type containing said synthetic construct.

The method may be further characterized by the step of introducing the synthetic construct into an organism or part thereof such that said synthetic construct is introduced into said at least one cell or tissue type.

In another aspect, the invention resides in a synthetic construct comprising a reporter polynucleotide fused in frame with a tandem repeat of identical codons, wherein said reporter polynucleotide

encodes a reporter protein, and wherein said synthetic construct is operably linked to one or more regulatory nucleotide sequences

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DETAILED DESCRIPTION

By "expressing said synthetic construct" is meant transcribing the synthetic construct such that mRNA is produced.

The term "synonymous codon" as used herein refers to a codon having a different nucleotide sequence to an existing codon but encoding the same amino acid as the existing codon.

By "isoaccepting transfer RNA" or "iso-tRNA" is meant one or more transfer RNA molecules that differ in their anticodon structure but are specific for the same amino acid.

The term "polynucleotide" as used herein designates mRNA, RNA, cRNA, cDNA or DNA.

The term "progenitor cell or tissue" as used herein refers to a cell or tissue that can gives rise to a particular cell or tissue in which codon preference is to be determined.

Throughout this specification, unless the context requires otherwise, the words "comprise", comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

The present invention is based the discovery that different stretches of identical codons fused respectively in frame with a polynucleotide can give rise to different levels of reporter protein expressed within a given cell type. Not wishing to be bound by theory, it is believed that a tandem series of identical codons may cause

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ribosome to pause during translation if the iso-tRNA corresponding to the identical codons is limiting. this regard, it is well known that ribosomal pausing leads to a failure to complete a nascent polypeptide chain and an uncoupling of transcription translation. Accordingly, the levels of reporter protein expressed in the different cells or tissues will be sensitive to the intracellular abundance of the iso-tRNA species corresponding to the identical codons and will therefore provide a direct correlation a cell's or tissue's preference for a given codon. This means for example, that if the levels of the reporter protein obtained in a cell or tissue type are lower with a synthetic construct having a tandem series of identical first codons compared to those expressed in the same cell or tissue type with a different synthetic construct having a tandem series of identical second codons, wherein the first codons are different to, but synonymous with, the second codons, then it can be deduced that the cell or tissue has a higher preference for the second codon relative to the first codon with respect to translation.

Suitably, the tandem repeat comprises least three identical codons. Preferably, the tandem identical codons, repeat comprises four codons. seven identical preferably five, six, or However, it will be appreciated that the number of identical codons utilized for the synthetic construct for example, on the regulatory may vary depending, sequences used to express the synthetic construct, the reporter polynucleotide employed, and the cell tissue under test. Accordingly, it is preferred that preliminary experiments be carried out to determine an optimal number of identical codons which is sensitive to the intracellular abundance of the corresponding iso-tRNA species, when expressed as part

synthetic construct. In this regard, too many identical codons may completely inhibit expression of the reporter protein whilst too few may not influence reporter protein expression at all.

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The tandem repeat may be fused at a location adjacent to, or within, the reporter polynucleotide. The location is preferably selected such that the tandem repeat interferes with translation of at least a portion of the reporter protein from which an activity can be measured. Preferably, the tandem repeat is located immediately upstream of the reporter polynucleotide.

The reporter polynucleotide may encode any levels mav be suitable protein whose determined indirectly such as by suitable assay. directly, or Suitable reporter polynucleotides include, but are not encoding **B**restricted to, polynucleotides alkaline firefly luciferase, galactosidase, phosphatase, chloramphenicol acetyltransferase (CAT), $\beta\text{-glucuronidase}$ (GUS), herbicide resistance genes such as the bialophos resistance (BAR) gene that confers to the herbicide BASTA, and resistance fluorescent protein (GFP). Assays for the activities associated with such proteins are well known by those Preferably, the reporter skill in the art. polynucleotide encodes GFP.

it will appreciated be that Of course reporter polynucleotides need not correspond to particular reporter gene encoding а full-length Accordingly, the invention protein. reporter polynucleotide sub-sequences contemplates encoding desired portions of the reporter protein. polynucleotide sub-sequence encodes a domain of the activity associated having an reporter protein therewith and preferably encodes at least 10, 20, 50,

100, 150, or 500 contiguous amino acids of the reporter protein.

The method of the invention is applicable to any suitable cell or tissue type. For example, the cell or tissue type may be of mammalian or plant origin. The cell or tissue type may be of suitable lineage. Suitable cell lines may include, example, CV-1 cells, COS cells, yeast spodoptera cells which are capable of being grown in The invention also contemplates cells which vitro. may be prokaryotic in origin.

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Suitable methods for isolating particular cells or tissues are well known to those of skill in For example, one can take advantage of one or more particular characteristics of a cell or tissue specifically isolate the cell or tissue from a heterogeneous population. Such characteristics include, but are not limited to, anatomical location of a tissue, cell density, cell size, cell morphology, cellular metabolic activity, cell uptake of ions such as Ca²⁺, K⁺, and H⁺ ions, cell uptake of compounds such as stains, markers expressed on the cell surface, fluorescence, membrane potential. protein and Suitable methods that may be used in this regard include surgical removal of tissue, flow cytometry techniques such as fluorescence-activated cell sorting (FACS), immunoaffinity separation (e.g., magnetic bead separation such as Dynabead™ separation), density separation (e.g., metrizamide, Percoll™, or Ficoll™ gradient centrifugation), cell-type specific and density separation.

In an alternate embodiment, progenitor cells or tissues may be used for initially introducing the synthetic construct. Any suitable progenitor cell or tissue may be used which gives rise to a particular

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cell or tissue of interest for which codon preference is to be ascertained. For example, a suitable progenitor cell may comprise an undifferentiated cell. In the case of a plant, a suitable progenitor cell may include, for example, a meristematic cell whereas a progenitor tissue may include, for example, a callus tissue.

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another embodiment, the In synthetic construct may be introduced firstly into an organism or part thereof before subsequent expression of the construct in a particular cell or tissue type. suitable organism is contemplated by the invention include unicellular as well as which may Exemplary multi-cellular cellular organisms. organisms include mammals and plants.

The construction of the synthetic construct may be effected by any suitable technique. example, in vitro mutagenesis methods may be employed which are well known to those of skill in the art. Suitable mutagenesis methods are described for example in the relevant sections of CURRENT PROTOCOLS MOLECULAR BIOLOGY (Ausubel, et al., eds.) (John Wiley and of MOLECULAR CLONING. 1997), Inc. Sons, et al., eds.) (Cold MANUAL (Sambrook, LABORATORY Spring Harbor Press, 1989), which are incorporated Alternatively, suitable methods herein by reference. for altering DNA are set forth, for example, in U.S. Patent Nos. 4,184,917, 4,321,365 and 4,351,901, which are incorporated herein by reference. Instead of in vitro mutagenesis, the synthetic polynucleotide may be synthesized de novo using readily available machinery. Sequential synthesis of DNA is described, for example, in U.S. Patent No 4,293,652, which is incorporated herein by reference. However, it should be noted that the present invention is not dependent on and not

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directed to any one particular technique for constructing the synthetic construct.

Regulatory nucleotide sequences which may be utilized to regulate expression of the synthetic polynucleotide include, but are not limited to, promoter, an enhancer, and a transcriptional Such regulatory sequences are well known terminator. to those of skill in the art. Suitable promoters which may be utilized to induce expression of the polynucleotides of the invention include constitutive promoters and inducible promoters.

Synthetic according constructs to the invention may be operably linked to one more or regulatory sequences in the form of an expression By "vector" is meant a nucleic acid molecule, vector. preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into which a synthetic nucleic acid sequence may be inserted or A vector preferably contains one or more cloned. unique restriction sites and may be capable autonomous replication in a defined host including a target cell or tissue or a progenitor cell or tissue thereof, or be integratable with the genome of the defined host such that the cloned sequence is Thus, by "expression vector" is meant reproducible. capable of directing the element autonomous synthesis of a protein. Such expression vectors are well known by practitioners in the art.

include a selection The vector may also marker such as an antibiotic resistance gene which can for selection of suitable used be transformants/transfectants. Examples of resistance genes include the nptII gene which confers resistance to the antibiotics kanamycin and

(Geneticin®) and the *hph* gene which confers resistance to the antibiotic hygromycin B.

The step of introducing the synthetic construct into a particular cell or tissue type, or into a progenitor cell or tissue thereof, or into an organism or part thereof for subsequent introduction. particular cell tissue, will or depending on the intended use and or species, and may involve lipofection, electroporation, micro-projectile bombardment infection by Agrobacterium tumefaciens or A rhizogenes, or protoplast fusion. Such methods are well known to those skilled in the art.

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Alternatively, the step of introduction may involve non-viral and viral vectors, cationic liposomes, retroviruses and adenoviruses such as, for example, described in Mulligan, R.C., (1993 Science 260 926-932) which is incorporated herein by reference. Such methods may include:

- Local application of the synthetic (i) nucleic acid sequence by injection (Wolff et al., 1990, Science 247 1465-1468, which is incorporated reference), surgical implantation, herein by instillation or any other means. This method may also used in combination with local application by injection, surgical implantation, instillation or any other means, of cells responsive to the reporter protein encoded by the synthetic construct. method may also be used in combination with local implantation, application by injection, surgical instillation or any other means, of another factor or factors required for the activity of said reporter protein.
- (ii) General systemic delivery by injection of DNA, (Calabretta et al., 1993, Cancer Treat. Rev. 19 169-179, which is incorporated herein

by reference), or RNA, alone or in combination with liposomes (Zhu et al., 1993, Science 261 209-212, which is incorporated herein by reference), viral capsids or nanoparticles (Bertling et al., 1991, 13 390-405, Biotech. Appl. Biochem. which is reference) herein by or any other incorporated Improved targeting might be mediator of delivery. linking the synthetic construct to achieved by so-called "magic bullet" targeting molecule (the approach employing for example, an antibody), or by local application by injection, surgical implantation any other means, of another factor or factors required for the activity of the protein produced from said synthetic construct, or of cells responsive to said reporter protein.

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Injection or implantation or delivery (iii) by any means, of cells that have been modified ex vivo by transfection (for example, in the presence of calcium phosphate: Chen et al., 1987, *Mole*. Cell Biochem. 7 2745-2752, or of cationic lipids 20 polyamines: Rose et al., 1991, BioTech. 10 520-525, which articles are incorporated herein by reference), electroporation (Shigekawa injection, infection, al., 1988, BioTech. 6 742-751, which is incorporated any other so herein by reference) or way 25 increase the expression of said synthetic construct in The modification may be mediated by those cells. bacteriophage, cosmid, viral (such plasmid, adenoviral or retroviral; Mulligan, 1993, Science 260 926-932; Miller, 1992, Nature 357 455-460; Salmons et 30 al., 1993, Hum. Gen. Ther. 4 129-141, which articles reference) or other incorporated herein by such vectors, or other agents of modification liposomes (Zhu et al., 1993, Science 261 209-212, which is incorporated herein by reference), viral 35

capsids or nanoparticles (Bertling et al., 1991, Biochem. 13 390-405, Biotech. Appl.which is incorporated herein by reference), or any mediator of modification. The use of cells as a delivery vehicle for genes or gene products has been described by Barr et al., 1991, Science 254 1507-1512 and by Dhawan et al., 1991, Science 254 1509-1512, which articles are incorporated herein by reference. Treated cells may be delivered in combination with any nutrient, growth factor, matrix or other agent that will promote their survival in the treated subject.

Advantageously, the relative preference for different codons may be determined by comparing the respective activities of the reporter protein in a given cell or tissue type. One of ordinary skill in the art will thereby be able to determine a relative codon preference table for the cell or tissue type.

The invention further contemplates cells or tissues containing therein the synthetic construct of the invention, or alternatively, cells or tissues produced from the method of the invention.

The invention is further described with reference to the following non-limiting examples.

25 EXAMPLE 1

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Construction of expression vectors for determining relative codon preferences in mammalian cells.

Synthetic gfp genes will be constructed in which a single artificial start codon (ATG) followed by a stretch of five identical codons is fused in frame immediately upstream of a gfp coding sequence. A reverse oligonucleotide primer (SEQ ID NO:180; sequence complementary to the termination codon for GFP, is underlined), and a suite of forward

oligonucleotide primers (SEQ ID NO: 121 through 179; first codon of underlined) will be the GFP, is synthesized and used for PCR amplification humanized gfp gene (SEQ ID NO:119) (GIBCO) as template Tag DNA polymerase (Amplification parameters: 95°C/30 sec; 52°C/30 sec; 72°C/1 min; 30 cycles). amplified fragments will have nucleic acid sequences and deduced amino acid sequences as shown in SEQ ID NO:1 through 120. In summary, the synthetic fragments contain an artificial start codon followed by a tandem repeat of five identical codons specific for a given immediately The tandem repeat iso-tRNA species. second codon of the The precedes the gfp gene. synthetic fragments by SEQ ID NO and encoded tandem repeat are presented in the TABLE 1.

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TABLE 1. Synthetic fragments and tandem repeats encoded thereby.

	neoded energy:		
SEQ ID NO	Tandem repeat	SEQ ID NO	Tandem repeat
1	Ala (GCA) x 5	61	Leu (CTC) x 5
3	Ala (GCC) x 5	63	Leu (CTG) x 5
5	Ala (GCG) x 5	65	Leu (CTT) x 5
7,	Ala (GCT) x 5	67	Leu (TTA) x 5
9	Arg (AGA) x 5	69	Leu (TTG) x 5
11	Arg (AGG) x 5	71	Lys (AAA) x 5
13	Arg (CGA) x 5	73	Lys (AAG) x 5
15	Arg (CGC) x 5	75	Phe (CTT) x 5
17	Arg (CGG) x 5	77	Phe (TTC) x 5
19	Arg (CGT) x 5	79	Pro (CCC) x 5
21	Asn (AAC) x 5	81	Pro (CCG) x 5
23	Asn (AAT) x 5	83	Pro (CCT) x 5
25	Asp (GAC) x 5	85	Pro (CGA) x 5
27	Asp (GAT) x 5	87	Ser (AGC) x 5
29	Cys (TGC) x 5	89	Ser (AGT) x 5

31	Cys (TGT) x 5	91	Ser (TCA) x 5
33	Gln (CAA) x 5	93	Ser (TCC) x 5
35	Gln (CAG) x 5	95	Ser (TCG) x 5
37	Gly (GAA) x 5	97	Ser (TCT) x 5
39	Glu (GAG) x 5	99	Thr (ACA) x 5
41	Gly (GGA) x 5	101	Thr (ACC) x 5
43	Gly (GGC) x 5	103	Thr (ACG) x 5
45	Gly (GGG) x 5	105	Thr (ACT) x 5
47	Gly (GGT) x 5	107	Trp (TGG) x 5
49	His (CAC) x 5	109	Tyr (TAT) x 5
51·	His (CAT) x 5	111	Val (GTA) x 5
53	Ile (ATA) x 5	113	Val (GTC) x 5
55	Ile (ATC) x 5	115	Val (GTG) x 5
57	Ile (ATT) x 5	117	Val (GTT) x 5
59	Leu (CTA) x 5	119	control

The amplified fragments will be cloned between the EcoRI and KpnI sites of the mammalian expression vector pCDNA3 containing SV40 ori (Invitrogen) and the CMV promoter.

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EXAMPLE 2

Transfection of Cos-1 cells

plates will cells in 6-well Cos-1 above expression transfected with 2 μq of the 10 using lipofectamine (Gibco). GFP constructs be analyzed 36 hrs after fluorescence will Synthetic gfp mRNA expression transfection. transfected cells will also be tested by 15 transcriptase PCR.

EXAMPLE 3

Confocal microscopy

Transfected CV-1 cells can be examined using a Bio-Rad MRC-600 laser-scanning confocal microscope

equipped with a krypton-argon laser and filter sets suitable for the detection of fluorescein and Texas red dyes (Bio-Rad KlyK2), and a Nikon 603 PlanApo numerical aperture 1.2 water-immersion objective. Dual-channel confocal images and video montages of the transfected cells can be suitably composed using ADOBE PhotoShop.

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. Those of skill in the art will appreciate that, in light of the present disclosure, numerous modifications and changes may be made in the particular embodiments exemplified without departing from the scope of the invention.

SEQUENCE LISTING

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5	<120> Polynucleotide and Method	
	<130> codon optimization	
10	<140> PPXXXX <141> 1999-01-08	
-	<160> 180	
15	<170> PatentIn Ver. 2.0	
	<210> 1 <211> 732 <212> DNA <213> Artificial Sequence	
20	<220> <223> Description of Artificial Sequence: Ala(GCA)5GFP	
25	<220> <221> CDS <222> (1)(732)	
30	<400> 1 atg agc agc agc agc agc agg gag gag ctg ttc act ggc gtg Met Ser Ser Ser Ser Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 1 5 10 15	
35	gtc cca att ctc gtg gaa ctg gat ggc gat gtg aat ggg cac aaa ttt 96 Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe 20 25 30	
	tct gtc agc gga gag ggt gaa ggt gat gcc aca tac gga aag ctc acc 144 Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 35 40 45	
40	ctg aaa ttc atc tgc acc act gga aag ctc cct gtg cca tgg cca aca 192 Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr 50 55 60	
45	ctg gtc act acc ttc tct tat ggc gtg cag tgc ttt tcc aga tac cca 240 Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro 65 70 75 80	
50	gac cat atg aag cag cat gac ttt ttc aag agc gcc atg ccc gag ggc 288 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 85 90 95	
55	tat gtg cag gag aga acc atc ttt ttc aaa gat gac ggg aac tac aag 336 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys 100 105 110	
	acc cgc gct gaa gtc aag ttc gaa ggt gac acc ctg gtg aat aga atc 384 Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile 115 120 125	
60	gag ctg aag ggc att gac ttt aag gag gat gga aac att ctc ggc cac 432 Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 130 135 140	
65	aag ctg gaa tac aac tat aac tcc cac aat gtg tac atc atg gcc gac 480 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp 145 150 155 160	
70	aag caa aag aat ggc atc aag gtc aac ttc aag atc aga cac aac att 528 Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile	

165 170 gag gat gga tcc gtg cag ctg gcc gac cat tat caa cag aac act cca Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro 5 180 185 atc ggc gac ggc cct gtg ctc ctc cca gac aac cat tac ctg tcc acc 624 Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 10 672 cag tot goo otg tot aaa gat ooc aac gaa aag aga gac cac atg gto Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val 215 15 720 ctg ctg gag ttt gtg acc gct gct ggg atc aca cat ggc atg gac gag Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu 732 ctg tac aag tga 20 Leu Tyr Lys <210> 2 <211> 243 <212> PRT 25 <213> Artificial Sequence Met Ser Ser Ser Ser Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 30 Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe 20 25 30 Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 35 40 4535 Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr 50 55 60 40 Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro 65 70 75 80 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 45 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile 50 Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 55 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 60 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro 180 185 190Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 65 200 Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val 70

Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu

	225					230					235					240	
	Leu T	yr I	Lys														
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7.0	atc Ile	ggc Gly	gac Asp 195	ggc Gly	cct	gtg Val	cto Leu	cto Leu 200	Pro	gac Asp	aac Asn	cat His	tac Tyr 205	Leu	tco Ser	acc Thr	624
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50	aag caa aag aat ggc atc aag gtc aac ttc aag atc aga cac aac att 529 Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 165 170 175	3
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65	ctg ctg gag ttt gtg acc gct gct ggg atc aca cat ggc atg gac gag 72 Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu 235 240	
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	Glu	Asp	Gly	Ser 180	Val	Gln	Leu	Ala	Asp 185	His	Tyr	Gln	Gln	Asn 190	act Thr	Pro	576
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J			atg Met														288
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135

70

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15			aag Lys														432
15	aag Lys 145	ctg Leu	gaa Glu	tac Tyr	aac Asn	tat Tyr 150	aac Asn	tcc Ser	cac His	aat Asn	gtg Val 155	tac Tyr	atc Ile	atg Met	gcc Ala	gac Asp 160	480
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65					85					90)				95		
70	_			100)				105	5				110)	Lys	
, 🗸	Thi	r Ar	g Ala	a Glu	ע Val	Lys	Phe	e Glu	ı Gly	y Asp	Thi	. Lei	ı Val	l Ası	n Ar	J Ile	

115 120 Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 5 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 10 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro 15 Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 200 Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val 20 Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 25 <210> 25 <211> 732 <212> DNA 30 <213> Artificial Sequence <223> Description of Artificial Sequence: Asp(GAC)5GFP 35 <220> <221> CDS <222> (1)..(732) <400> 25 48 40 atg gac gac gac gac agc aag ggc gag gaa ctg ttc act ggc gtg Met Asp Asp Asp Asp Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 96 gtc cca att ctc gtg gaa ctg gat ggc gat gtg aat ggg cac aaa ttt 45 Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe tct gtc agc gga gag ggt gaa ggt gat gcc aca tac gga aag ctc acc Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 144 50 ctg aaa ttc atc tgc acc act gga aag ctc cct gtg cca tgg cca aca Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr 192 55 ctg gtc act acc ttc tct tat ggc gtg cag tgc ttt tcc aga tac cca 240 Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro
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75
80 gac cat atg aag cag cat gac ttt ttc aag agc gcc atg ccc gag ggc 288 60 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly tat gtg cag gag aga acc atc ttt ttc aaa gat gac ggg aac tac aag 336 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys 65 105 acc cgc gct gaa gtc aag ttc gaa ggt gac acc ctg gtg aat aga atc Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile

120

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55	aag ctg gaa tac aac tat aac tcc cac aat gtg tac atc atg gcc gac Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp 145 150 155 160	
60	aag caa aag aat ggc atc aag gtc aac ttc aag atc aga cac aac att 528 Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 165 170 175	
c. F	gag gat gga tcc gtg cag ctg gcc gac cat tat caa cag aac act cca 576 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro 180 185 190	
65	atc ggc gac ggc cct gtg ctc ctc cca gac aac cat tac ctg tcc acc 624 Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 195 200 205	
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		Gly	195					200					205				
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:

and the second second second

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Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile

70

Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 135 130 5 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 10 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 15 200 Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val 20 Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 25 <210> 51 <211> 732 <212> DNA <213> Artificial Sequence 30 <223> Description of Artificial Sequence: His(CAT)5GFP <220> 35 <221> CDS <222> (1)..(732) <400> 51 atg cat cat cat cat agc aag ggc gag gaa ctg ttc act ggc gtg Met His His His His Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 48 40 gtc cca att ctc gtg gaa ctg gat ggc gat gtg aat ggg cac aaa ttt Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe 45 tct gtc agc gga gag ggt gaa ggt gat gcc aca tac gga aag ctc acc 144 Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 50 192 ctg aaa ttc atc tgc acc act gga aag ctc cct gtg cca tgg cca aca Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr ctg gtc act acc ttc tct tat ggc gtg cag tgc ttt tcc aga tac cca Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro 55 gac cat atg aag cag cat gac ttt ttc aag agc gcc atg ccc gag ggc Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 288 60 85 tat gtg cag gag aga acc atc ttt ttc aaa gat gac ggg aac tac aag 336 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys 65 105 acc cgc gct gaa gtc aag ttc gaa ggt gac acc ctg gtg aat aga atc 384 Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile 120 70 gag ctg aag ggc att gac ttt aag gag gat gga aac att ctc ggc cac 432

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Leu Tyr Lys

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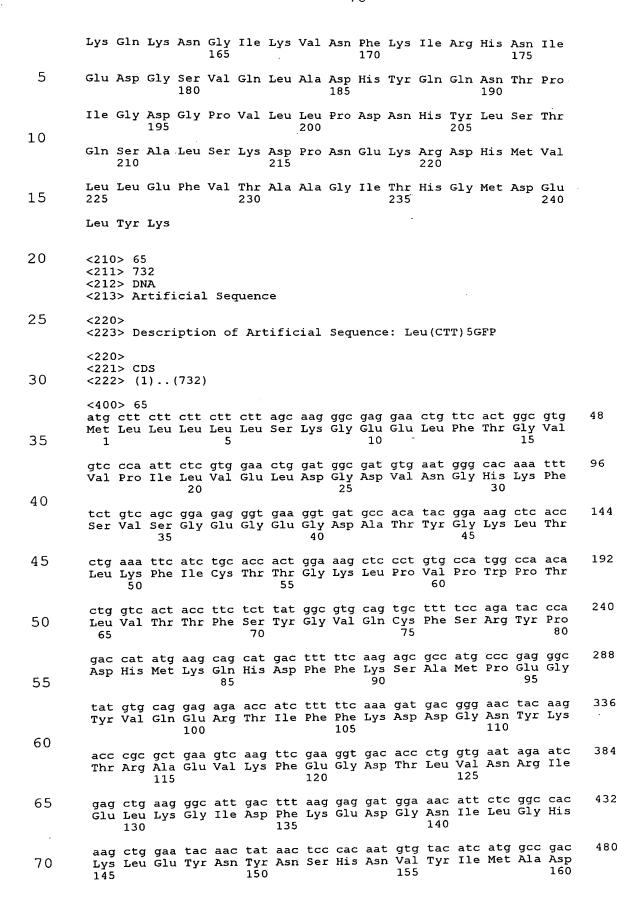
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المراجعة المحاجبين المحاجبين المحاجبين

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)



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3	gag Glu																576
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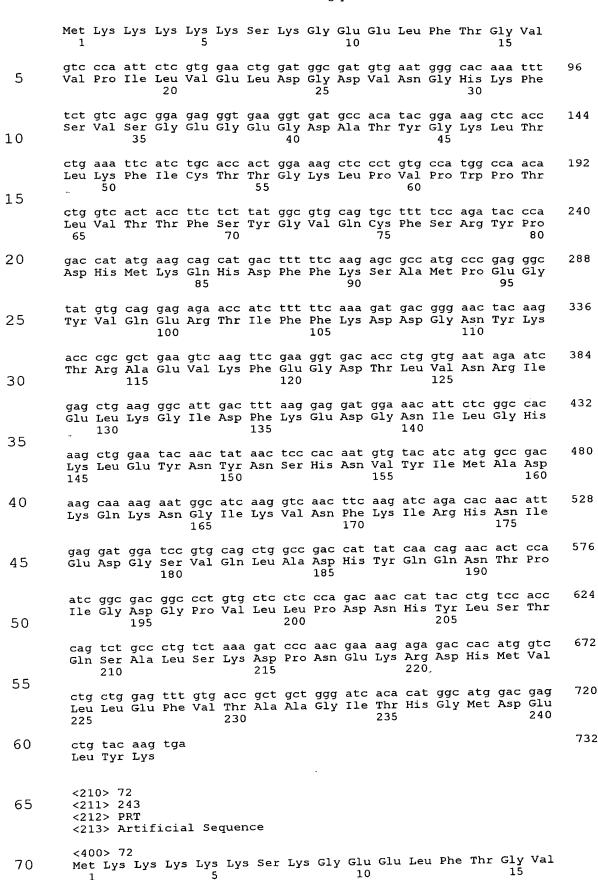
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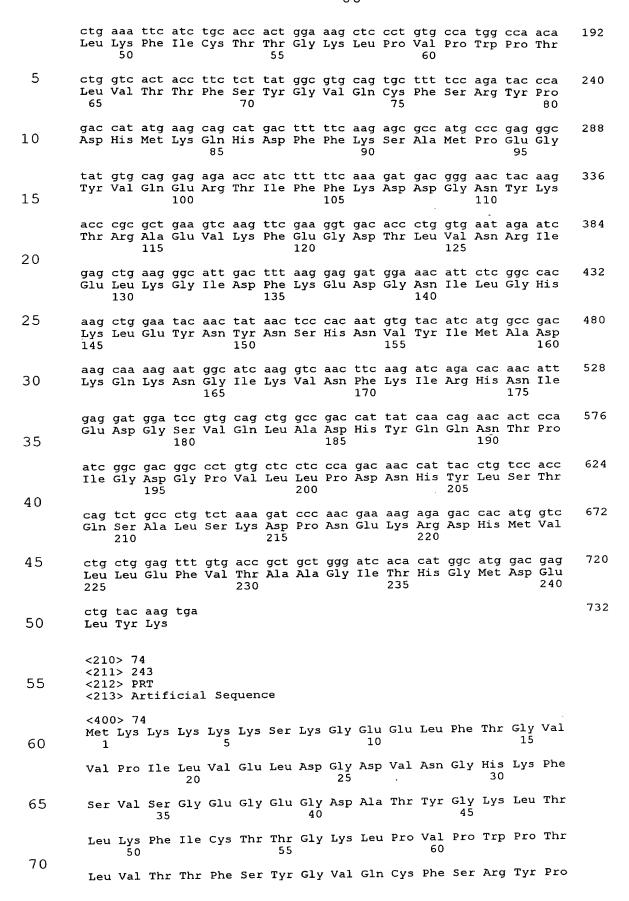


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Carrier Congress Constraints

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70 65 75 80 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 5 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile 10 Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 15 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp 145 - 150 155 160 Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 165 170 20 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 25 Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val 30 Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 35 <210> 75 <211> 732 <212> DNA <213> Artificial Sequence 40 <223> Description of Artificial Sequence: Phe(CTT)5GFP <220> 45 <221> CDS <222> (1)..(732) <400> 75 atg ttg ttg ttg ttg agc aag ggc gag gaa ctg ttc act ggc gtg 48 Met Leu Leu Leu Leu Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 50 gtc cca att ctc gtg gaa ctg gat ggc gat gtg aat ggg cac aaa ttt Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe 96 55 tct gtc agc gga gag ggt gaa ggt gat gcc aca tac gga aag ctc acc Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 60 ctg aaa ttc atc tgc acc act gga aag ctc cct gtg cca tgg cca aca 192 Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr 50 ctg gtc act acc ttc tct tat ggc gtg cag tgc ttt tcc aga tac cca Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro 240 65 gac cat atg aag cag cat gac ttt ttc aag agc gcc atg ccc gag ggc 288 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 70 90 85

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Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro

180

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	Lys Gln		165			٠	-	170					175		
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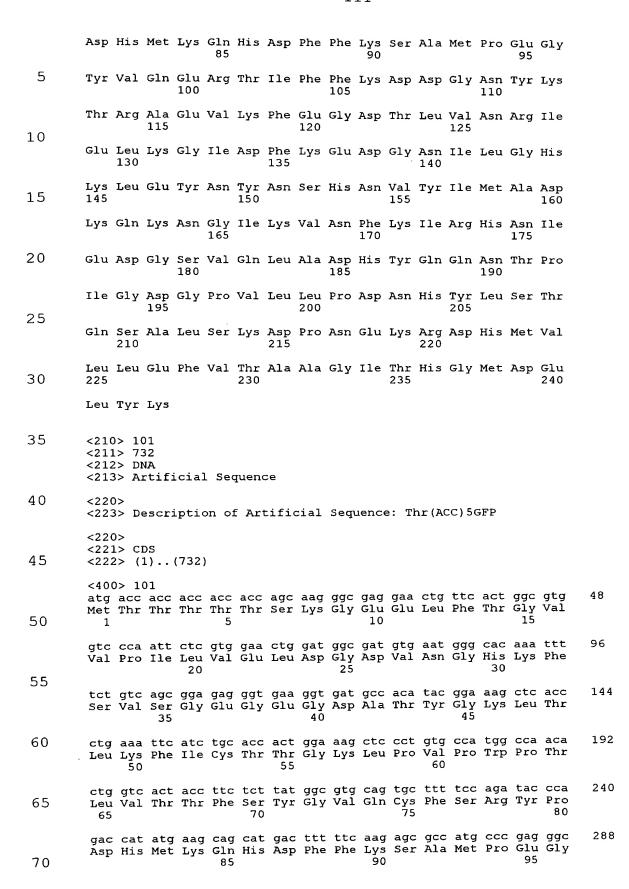
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N.S.

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135

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ς,

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and the control of th

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65 70 75 80 5 Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly 10 Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile 120 15 Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His 135 Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp 20 Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile 25 Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro 185 Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr 200 30 Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu 35 230 Leu Tyr Lys 40 <210> 113 <211> 732 <212> DNA <213> Artificial Sequence 45 <223> Description of Artificial Sequence: Val(GTC)5GFP <220> <221> CDS 50 <222> (1)..(732) 48 atg gtc gtc gtc gtc agc aag ggc gag gaa ctg ttc act ggc gtg Met Val Val Val Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val 55 10 gtc cca att ctc gtg gaa ctg gat ggc gat gtg aat ggg cac aaa ttt Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe 20 25 30 20 60 tct gtc agc gga gag ggt gaa ggt gat gcc aca tac gga aag ctc acc 144 Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr 192 ctg aaa ttc atc tgc acc act gga aag ctc cct gtg cca tgg cca aca 65 Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr 55 ctg gtc act acc ttc tct tat ggc gtg cag tgc ttt tcc aga tac cca Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro 65 70 75 80 70

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Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile

to the time of the contract of

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165 170 175

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40	tct tat Ser Tyr 65	ggc Gly	gtg Val	cag Gln	tgc Cys 70	ttt Phe	tcc Ser	aga Arg	tac Tyr	cca Pro 75	gac Asp	cat His	atg Met	aag Lys	cag Gln 80	240
40	cat gac His Asp	ttt Phe	ttc Phe	aag Lys 85	agc Ser	gcc Ala	atg Met	ccc Pro,	gag Glu 90	ggc Gly	tat Tyr	gtg Val	cag Gln	gag Glu 95	aga Arg	288
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